

Chapter 12

Roles of Rhizospheric Processes and Plant Physiology in Applied Phytoremediation of Contaminated Soils Using *Brassica* Oilseeds

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Abstract The current chapter reviews in detail significant physiological mechanisms of metal accumulating *Brassica* species and discusses rhizospheric processes and soil management, including the role of soil amendments such as chelators in enhancing the uptake of toxic metals, focusing on their roles in phytoremediation of contaminated sites worldwide, in addition to presenting an overview of the field of phytoremediation, including its merits and shortcomings. Recent progress towards the use of oilseed *Brassica* species in field-based studies is also discussed.

Keywords *Brassica* oilseeds • Contaminated soils • Phytoremediation • Rhizospheric processes

12.1 Introduction

Heavy metal contamination of soil and water is a major threat to human and ecosystem health, making the cleanup of metal-contaminated sites a high priority (Ensley 2000). The persistent nature of heavy metal contaminants in the environment has meant that the most commonly used methods of cleanup of contaminated soil are excavation and removal, incineration, and chemical treatment (Pilon-Smits 2005). These methods are not only damaging to the environment, but are also expensive, making it largely impossible for developing nations to adopt such methods (Rajakaruna et al. 2006). The United States spends \$6–8 billion per year on environmental cleanup, and globally the cost is \$25–50 billion a year (Tsao 2003; Pilon-Smits 2005).

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Recently, much research has been conducted on the use of hyperaccumulator plants in the cleanup of metal-contaminated sites (Cunningham and Ow 1996; Pilon-Smits and Freeman 2006). Hyperaccumulators take up high concentrations of heavy metals from the soil and translocate them into above-ground biomass at concentrations exceeding, in most cases, 0.1% total dry leaf tissue mass ($>1,000 \mu\text{g metal g}^{-1}$ dry leaf tissue) (Baker et al. 2000). The threshold values set for hyperaccumulation are element-dependent: Mn and Zn hyperaccumulators accumulate $>10,000 \mu\text{g g}^{-1}$ dry mass, hyperaccumulators of As, Co, Cu, Ni, Se and Pb accumulate $>1,000 \mu\text{g g}^{-1}$, and hyperaccumulators of Cd accumulate $>100 \mu\text{g g}^{-1}$ (Reeves and Baker 2000). Hyperaccumulators of Al (Jansen et al. 2002), B (Babaoglu et al. 2004) and Fe (Rodríguez et al. 2005) have also been identified. The “elemental defense” hypothesis (Boyd 2004, 2009) speculates that hyperaccumulating species utilize the metals for defense against natural enemies, such as herbivores and pathogens (Fones et al. 2010; Quinn et al. 2010; Rascio and Navari-Izzo 2011; Strauss and Boyd 2011). Hyperaccumulators are often restricted to metal-enriched soils such as those found on serpentinite outcrops and other metalliferous rocks (Reeves 2002) and are worthy of careful study and conservation (Whiting et al. 2004; Rajakaruna and Boyd 2008; Harrison and Rajakaruna 2011).

While some plants can tolerate metalliferous soils by excluding metals (*i.e.* metal excluders) through binding them to exuded organic acids or sequestering them within the roots, hyperaccumulators are distinguished from such excluders by their high rate of metal uptake and translocation and their ability to accumulate and detoxify these extraordinarily high levels of metals in their shoots, especially leaves (Rascio and Navari-Izzo 2011). Recent research shows that the process of hyperaccumulation results from the overexpression of genes which encode transmembrane transporters (Rascio and Navari-Izzo 2011). In this chapter, we will explore the processes of hyperaccumulation from the perspectives of internal plant physiological processes and soil-plant relations, and their relevance to developing sound metal cleanup technologies.

Although the currently documented >450 metal hyperaccumulators occur in over 34 different families, 25% of hyperaccumulating species are in the family Brassicaceae (Rascio and Navari-Izzo 2011), the best known of which are in the genera *Alyssum* and *Noccaea* (formerly, *Thlaspi*) (Verbruggen et al. 2009). *Arabidopsis thaliana*, also from Brassicaceae, has been used extensively as a model organism for plant-based research (Bevan and Walsh 2005), including metal tolerance and accumulation (Cho et al. 2003). Given *A. thaliana*'s genome is mapped and there is a high level of sequence homology between it and its metal accumulating relatives, including *A. halleri* (Becher et al. 2004; Weber et al. 2004), *A. thaliana* has provided excellent means to explore the genetic basis for both metal tolerance and accumulation in related taxa, making Brassicaceae a model family for the study of metal tolerance and hyperaccumulation.

The use of hyperaccumulators to clean up toxic sites, *i.e.* phytoremediation, is fast gaining global recognition as a viable green technology to clean up metal contaminated sites (Pilon-Smits and Freeman 2006). Phytoremediation is based on the premise that plants which remove heavy metals from the soil and translocate

them to their above-ground biomass can then be harvested and disposed of through incineration or metal recovery, known as phytomining (Brooks et al. 1998). Phytoremediation is a much more environmentally friendly and, importantly, a less expensive alternative to traditional excavation-based methods of cleanup. However, there are several limitations to wide-scale implementation of this technology (Angle et al. 2001; Pilon-Smits 2005), including the length of time the cleanup might take and the fact that soil environments are complicated and can be volatile, and therefore the extent of uptake and cleanup harder to predict. Hence, more research, especially field experiments, needs to be done in order to advance this new technology toward widespread application.

12.2 Internal Plant Physiological Mechanisms

To avoid metal toxicity, plants employ internal physiological mechanisms of metal detoxification. Much research has been done in this area (see Kramer 2010), but much remains to be done. Generally, detoxification involves chelation of the metal cation by ligands or organic acids, or vacuolar or cell wall sequestration away from metabolic sites in the cytoplasm, usually within localized areas in the shoot (Baker et al. 2000; Lasat and Kochian 2000; Salt et al. 2000; Broadhurst et al. 2004; Chaney et al. 2007; Memon and Schroder 2009). The breadth and scope of these mechanisms are detailed and complex.

12.2.1 Chelation, Ion Uptake and Metal Loading

Both apoplastic and symplastic pathways are involved in the transport of inorganic ions. The three main steps in inorganic ion transport in the symplastic pathways are active transport of metals across root membranes; entry of metals into symplast during translocation from root to shoot; and chelation and sequestration of metals into specific compartments in the leaves (Maestri et al. 2010). Chelation of metals within the plant allows for xylem loading and transport, as well as for sequestration. These mechanisms involve many metal-specific chelators—*i.e.* ligands and organic acids—many of which have only begun to be studied or have not yet been characterized. Research on chelators and transporters mostly focuses on their genetic basis in order to determine how they work and how genetic engineering might help in producing more commercially desirable hyperaccumulators for phytoremediation (Pilon-Smits and LeDuc 2009). The purposes of the proceeding sections are not to delve into the genetic basis of metal tolerance and hyperaccumulation (see Pollard et al. 2002; Yang et al. 2005a, b for detailed reviews), but discuss general trends with respect to internal ion transport and detoxification of heavy metals in hyperaccumulators.

The role of chelators in hyperaccumulation is to form complexes with heavy metal ions. This can serve the function of aiding in transport, or it can be the terminus of the ion, leading to sequestration in the shoot of the plant. Metallothioneins and phytochelatins are two classes of chelators involved in metal accumulation (Cobbett and Goldsbrough 2002). These are sulfur-donor ligands which are better electron donors than oxygen and therefore form high-stability complexes with first-row transition metals (Baker et al. 2000). The role of genes in transporting metals across electrochemical gradients and in translocation in general is clearly important. The overexpression of certain genes in hyperaccumulators leads to greater amounts of chelators and transporters within the plant (Verbruggen et al. 2009). Enhanced root uptake of Zn, for example, seems to be driven by overexpression of members of the ZIP family—Zn- and Fe-regulated transporter proteins (Verbruggen et al. 2009; Rascio and Navari-Izzo 2011). In Se hyperaccumulators like *Stanleya pinnata* (Brassicaceae; Zhu et al. 2009), uptake seems to be correlated with the presence of a high-affinity SO_4^{2-} transporter in the plant, which is more expressed in *S. pinnata* than in non-hyperaccumulating congeners (Rascio and Navari-Izzo 2011). Further, in non-hyperaccumulators, many members of the ZIP family are expressed only under conditions of Zn deficiency, whereas they seem to be expressed independent of Zn supply in hyperaccumulators (Verbruggen et al. 2009). Further, reduced vacuolar sequestration of Zn in the root cells of *Thlaspi caerulescens* (now *Noccaea caerulescens*, see Koch and Mummenhoff 2001 and Koch and Al-Shehbaz 2004 for the taxonomic revisions; however, we maintain previous nomenclature in the chapter) and enhanced activity of the gene HMA4 (P-type metal ATPase) facilitated active xylem loading (Verbruggen et al. 2009).

Among the hyperaccumulation-related chelators resulting from overexpressed genes are metallothioneins (Clemens 2001), which are cysteine-rich, low-molecular-weight, metal-binding proteins that can bond with different metals; however, they are primarily implicated in metal homeostasis in mammals (Maestri et al. 2010) and their role in plant metal uptake and sequestration is still largely unclear. There are several families of metal transporters that have been implicated in metal uptake and translocation: Plasma membrane and tonoplast transporters involved in uptake, tonoplast transporters for remobilization from vacuoles, transporters for xylem loading, and endomembrane transporters (Maestri et al. 2010). Different organic acids and ligands have been found to be associated with various metals in distinct parts of different plants; for example, in *T. caerulescens*, most Zn in roots was associated with histidine, while in shoots it was associated with organic acids (Verbruggen et al. 2009). Also in *T. caerulescens*, Cd in the leaves was found to be bound with sulfur ligands (Verbruggen et al. 2009). In *Arabidopsis halleri*, Zn was mostly stored in the vacuoles of mesophyll, while in *T. caerulescens* it was in the vacuoles of the epidermal cells (Verbruggen et al. 2009). In both cases, vacuolar sequestration of Zn in the leaves was thought to be the main mechanism of detoxification and this is a distinguishing trait between hyperaccumulators and non-hyperaccumulators. In general, the inside of the vacuole is an environment conducive to formation of metal-organic acid complexes because it is acidic; in fact it was observed that within the vacuoles of *T. caerulescens* and *A. halleri*, a large pool of malate led to the

formation of Zn-malate complexes, in mesophyll vacuoles of *A. halleri* and in the epidermal cell vacuoles in *T. caerulescens* (Verbruggen et al. 2009).

Several studies have pointed to the amino acid histidine as playing an important role in hyperaccumulation (Baker et al. 2000; Verbruggen et al. 2009; Richau et al. 2009; Kramer 2010). Baker et al. (2000) found that the Ni as a histidine complex has a higher stability than Ni complexed with any other amino or organic acid. Histidine forms stable complexes with Ni, Zn, and Cd (Verbruggen et al. 2009). Dose-dependent increases in histidine occurred in the xylem sap of hyperaccumulator *Allysum lesbiacum* but not in the congeneric, non-hyperaccumulator *A. montanum* (Verbruggen et al. 2009). Histidine also plays a key role as a chelator in the tolerance as well as the high rate of translocation of Ni in *A. lesbiacum* (Kramer 2010). However, Chaney et al. (2007) reported that *Thlaspi* taxa with histidine-producing genes did not increase Ni accumulation, and that increased histidine did not make normally non-accumulating plants hyperaccumulate Ni. Therefore, the role and importance of histidine in hyperaccumulation may vary depending on other factors than simply the presence of histidine alone. Another organic acid which is associated with Ni in aqueous extracts of leaf material is malate (Baker et al. 2000). Malate has also been implicated in Zn tolerance, binding to Zn upon uptake, carrying ions to the vacuole where they are complexed by terminal acceptors, perhaps S-containing mustard oils in *T. caerulescens*, after which the malate is released to transport more Zn (Baker et al. 2000). Many Ni and Zn hyperaccumulators have high basal levels of malate and citrate, which may increase with increasing levels of Ni and Zn in the shoot, implying their role in detoxification (Chaney et al. 2007). *Thlaspi caerulescens* has also been shown to possess an association between malate and Cd (Chaney et al. 2007).

12.2.2 Translocation

Salt et al. (2000) reported most cellular Ni in the hyperaccumulator *Thlaspi goesingense* is associated with organic acids. Most organic acids are vacuolar; therefore this supports the hypothesis of vacuolar localization of Ni in hyperaccumulators. Storage of Zn in leaf vacuoles has also been documented (Lasat and Kochian 2000); leaf cells in *T. caerulescens* were bathed in xylem solution with high levels of Zn, suggesting that reabsorption of Zn from xylem into leaf cell walls is an important component of hyperaccumulation in this species. As pointed out earlier, the major difference between hyperaccumulators and metal excluders seems to be that the hyperaccumulators translocate a significantly greater percentage of accumulated heavy metal ions to their shoots for sequestration in the leaves, whereas metal excluders, while still being able to contain large amounts of heavy metals, do so in the roots (Lasat and Kochian 2000; Broadhurst et al. 2004; Memon and Schroder 2009; Richau et al. 2009; Salt et al. 2000; Verbruggen et al. 2009). Salt et al. (2000) report that in hydroponic solution, root Ni concentration in non-hyperaccumulator *T. arvense* was higher than in hyperaccumulator *T. goesingense*, while the reverse was true of shoot Ni concentration. *Thlaspi arvense* also displayed

lower shoot biomass than *T. goesingense* upon Ni exposure. However, both plants translocated Ni to the shoot at the same rate when exposed to nontoxic Ni levels, highlighting the different mechanisms by which hyperaccumulators and non-hyperaccumulators cope with high levels of heavy metals (Salt et al. 2000).

12.2.3 Root Anatomy and Physiology

The first part of a plant to come into contact with the soil is the root. The question as to whether the root of a hyperaccumulator is structurally different than that of a non-accumulator may account for some of the differences in uptake of heavy metals. Mench et al. (2009) found that *Thlaspi caerulescens* develops a peridendodermal layer, a zone in the roots with thickened inner tangential cell walls which form a continuous layer, externally attached to the endodermis. They are located near the root tip, which may indicate a role in ion transport (Mench et al. 2009). Ion transport in the root was elaborated upon by Lasat and Kochian (2000), reporting that in hyperaccumulator *T. caerulescens* and excluder *T. arvense*, transport of Zn across root cell membranes is mediated by proteins with similar Zn affinities. However there might be a higher expression of Zn transporters in *T. caerulescens*, which could account for the deployment of more transporters to the root cell membrane in that species than in *T. arvense*. Richau et al. (2009) also compared Ni translocation patterns of *T. caerulescens* and *T. arvense*. They found that the high rate of Ni translocation in *T. caerulescens* compared to *T. arvense* seems to be dependent on two factors: greatly enhanced concentration of root histidine in *T. caerulescens* and a strongly decreased ability to accumulate histidine-bound Ni in root cell vacuoles in *T. caerulescens*. The concentration of free histidine in *T. caerulescens* was ten-fold higher than that in *T. arvense*, but only slightly higher in leaves, regardless of Ni exposure. Xylem loading of Ni was enhanced by exogenous histidine in *T. caerulescens* but not in *T. arvense*. These results suggest that root-to-shoot transport is constitutive at the species level for *T. caerulescens*, rather than simply enhanced Ni uptake (Richau et al. 2009). Shoot-derived tonoplast vesicles in *T. caerulescens* accumulate more Ni than root-derived ones, while the opposite is true in *T. arvense*. It is implied that chelation of Ni by histidine in the cytoplasm inhibits root vacuolar sequestration in *T. caerulescens* but not in *T. arvense*. Lower vacuolar sequestration of Zn in hyperaccumulator roots is also implicated in higher Zn accumulation in leaves. In addition, it was reported that in three hyperaccumulating *Alyssum* species, dose-dependent Ni exposure induced increases in histidine in xylem sap not seen in non-hyperaccumulators of Brassicaceae (Richau et al. 2009). However, for some non-hyperaccumulators (*A. montanum* and *Brassica juncea*) exogenously supplied histidine increased Ni tolerance and Ni xylem loading (Richau et al. 2009). The same was not true for *A. lesbiacum*, likely because of its five-fold higher (compared to *B. juncea*) root histidine concentration (Richau et al. 2009). Further, the formation of Ni-histidine complex inhibited the retention of Ni in root cell vacuoles of *T. caerulescens*

(Verbruggen et al. 2009). These findings suggest there are generally higher levels of free histidine in the roots of hyperaccumulators compared with those of non-hyperaccumulators and that greater levels of translocation occur in the hyperaccumulators as a result.

12.2.4 Metals Localization and Sequestration

Salt et al. (2000) evaluated Ni concentrations in hyperaccumulator *T. goesingense* compared with the non-accumulating *T. arvense*. Protoplasts were isolated from both species, and it was found that the ones from *T. goesingense* were more Ni-tolerant than those of *T. arvense*, suggesting a cellular mechanism of Ni tolerance in the leaves of the hyperaccumulator. It appears that Ni was localized within the vacuole of *T. goesingense*, as has also been shown for Cd and Zn (Salt et al. 2000).

Broadhurst et al. (2004) found that the majority of hyperaccumulated Ni in five *Alyssum* hyperaccumulator species grown in Ni-enriched soils is stored in either the leaf epidermal cell vacuoles or in the basal portions of the numerous stellate trichomes. In fact, the metal concentration in these trichome basal compartments was ~15–20% of dry weight, which is one of the highest concentrations to be reported in living plant tissue (Broadhurst et al. 2004). The authors also found that there was simultaneous and region-specific localization of high levels of Ni, Ca, and Mn within the trichomes, although the soils were only Ni-enriched and not Ca- or Mn-enriched. The region-specific localization of hyperaccumulated Ni, Ca, and Mn was found in all five *Alyssum* taxa, over a range of Ni concentrations (Broadhurst et al. 2004).

In another study, Ghasemi et al. (2009) measured Ni accumulation in the trichomes of the serpentine-endemic Ni-hyperaccumulator *Alyssum inflatum*. Using elemental analysis of the plants in their native habitat in Iran, they did not find that the trichomes accumulated any more Ni than the rest of the shoot. However, after treating plants with different concentrations of Ni in the growth medium and staining them with dimethylglyoxime (a stain specific for Ni), it was found that staining of the trichomes increased as the external dose of Ni increased. At lower Ni concentrations, the metal accumulated at the base of the trichomes; at higher Ni levels, accumulation extended to the rays and cell walls. Therefore, high levels of Ni can be accumulated in the trichomes of *A. inflatum*, and Ni accumulation as a whole can reflect Ni concentration in the shoot.

Galeas et al. (2006) looked at seasonal differences in the uptake of Se and S (chemically similar elements) in two hyperaccumulators, including *Stanleya pinnata*, over two growing seasons. The authors found that there were several stages of accumulation which depended on the time of year. In both hyperaccumulators tested, it was found that Se traveled from root to young leaves in the spring, then moved from aging leaves to flowers and seeds in the summer, and finally back to roots in autumn. These findings on seasonal translocation and

allocation of metals could have significant impacts on the application of phytoremediation methods.

In the preceding section we touched upon the depth and breadth of internal plant physiology of hyperaccumulators which is crucial for developing better practices of phytoremediation. The importance of the rhizosphere in uptake of metals in hyperaccumulators and its potential roles in the advancement of phytoremediation technologies are discussed in the following section.

12.3 Soil Environment-Confined Processes

12.3.1 *Chelation in the Rhizosphere*

The contrasts in root vs. shoot chelation and sequestration of heavy metals between hyperaccumulators and excluders presented in the previous section are not exclusive to internal plant physiology. Indeed, roots live in the soil, and therefore interact with soil and its myriad of components, including biota. In the previous section, we reported on studies which found that metal-tolerant excluders or non-hyperaccumulators tend to sequester heavy metals in their roots, while hyperaccumulators express much higher levels of translocation rates of these metals which are subsequently stored in the leaves. The same players in the processes of sequestration and transport—*i.e.* chelators such as histidine—also play a role in the initial uptake of metals from the soil environment. One hypothesis posits that roots of hyperaccumulators, which have a high ‘requirement’ for certain heavy metals, exude chelators into the rhizosphere in order to aid in uptake. Several studies have approached this hypothesis with varying results. Wenzel et al. (2003) conducted an experiment on the hyperaccumulator, *T. goesingense*, and two excluders, *Silene vulgaris* (Caryophyllaceae) and *Rumex acetosella* (Polygonaceae), growing on serpentine soil and found that organic ligand exudation may contribute to enhanced solubility and replenishment of metals in the rhizosphere of hyperaccumulators. They reported higher dissolved oxygen content (DOC) and Ni concentrations in water extracts of the rhizosphere of *T. goesingense* as well as decreased exchangeable Ni, which they attributed to excessive Ni uptake. They concluded that enhanced Ni solubility in the rhizosphere of *T. goesingense* was driven by the formation of Ni-organic acid complexes and ligand-induced dissolution of Ni-bearing minerals.

Salt et al. (2000), however, concluded that the speculation that Ni-specific chelators exuded by roots were responsible for greater uptake of Ni in *T. goesingense* was not supported because they were unable to identify any such high-affinity Ni-chelating compounds in the root exudate. In contrast, it was found that the root exudate of non-hyperaccumulator *T. arvense* actually contained higher levels of known Ni-chelators, histidine and citrate, than the root exudates of *T. goesingense* (at a Ni exposure rate of 25 mM over 48 h). Furthermore, the levels

of these Ni chelators in the root exudate of *T. arvense* increased upon Ni exposure, while the same compounds in the *T. goesingense* exudates remained at steady levels. Therefore, it was concluded that root-exuded histidine does not play a significant role in enhanced foliar accumulation of Ni by *T. goesingense*. These findings, however, suggest that the increased release of histidine by the non-accumulator *T. arvense* roots may be part of a Ni-detoxification strategy by chelating Ni in the rhizosphere, thereby reducing the activity of the Ni in the soil solution and decreasing its toxicity (Salt et al. 2000). Nevertheless, it was found that at elevated Ni concentrations, this mechanism was quickly overwhelmed, leading to plant toxicity. Puschenreiter et al. (2003) also concluded that increased DOC in the rhizosphere of the excluder species *T. arvense* alleviated phytotoxicity by formation of metal-organic compounds.

12.3.2 Importance of pH

The existing soil chemical and physical features of a site are vital in plant-rhizosphere interactions, and pH is likely the most important factor characterizing the soil environment of a plant (Rajakaruna and Boyd 2008). It affects a myriad of processes, some well-understood and some yet undiscovered; however understanding how pH affects hyperaccumulation potential is a critical step in developing sound phytoremediation technologies. pH has been shown to affect the metal extraction capacity of *T. caerulescens* for both Cd and Zn (Rascio and Navari-Izzo 2011; Wang et al. 2006). Wang et al. (2006) studied *T. caerulescens* in two soils with varying levels of Cd and Zn at six pH levels, and concluded that lowering the pH in both soils not only increased the soluble forms of both metals, but that *T. caerulescens* had the highest shoot biomass at the lowest pH (4.74) and the highest shoot metal concentration at the second-lowest pH (5.27). Kukier et al. (2004) conducted an experiment on the effect of pH on uptake of Ni and Co by hyperaccumulators *Alyssum corsicum* and *A. murale*. The plants were tested using three types of soil. Two of the soils—Quarry muck (Terric Haplohemist) and Wellend (Typic Epiaquoll)—came from a site in Port Colborne, Ontario, Canada that had been contaminated by a Ni refinery. The third soil, Brockman (Typic Xerochrepts), was a serpentine soil from Oregon, USA. Soils were treated with limestone to adjust the pH from highly acidic to mildly alkaline. Both the Quarry muck and the Wellend soils had a relatively low initial pH (5.66 and 5.24, respectively), while the Brockman serpentine had a slightly higher initial pH (6.30). Both *Alyssum* species grown in the industrially contaminated soils showed increased Ni shoot concentrations with increased soil pH; the highest fraction of total soil Ni extracted was 6.3% from the Quarry muck, followed by Wellend (4.7%) and Brockman (0.84%) at pH 7.3, 7.7, and 6.4, respectively (Kukier et al. 2004). Interestingly, increased Ni uptake occurred despite a decrease in water-soluble Ni in the soil, which is contrary to what is generally seen with agricultural crops (Kukier et al. 2004). There was a slight decrease in shoot Ni concentration

with increased pH in the serpentine soil. Cobalt concentrations increased with increased pH in the Quarry muck, yet decreased in the Wellend and Brockman soils. The differences in uptake of Ni and Co from the different soils with varying pH by the *Alyssum* taxa was thought to be related to differences in percent organic matter and Fe content of soils (Kukier et al. 2004). These conclusions, not surprisingly, point to other important aspects of the soil environment, not directly related to pH, which can also affect plant uptake of heavy metals. However, the study shows a general trend toward what might be expected, which is that increased pH generally leads to increased uptake of heavy metals by hyperaccumulators. In fact, Maestri et al. (2010) suggest that root exudates such as organic acids and phytosiderophores may play a role in metal mobilization and uptake because of their ability to acidify, not necessarily to chelate metals. However, root exudates from *T. caerulescens* did not increase Cd and Zn mobility compared with those of non-accumulators (Maestri et al. 2010). It is clear that the chemical environment of the rhizosphere plays an important role in metal bioavailability and mobility in the soil. For example, Cd and Zn uptake by *T. caerulescens* has been shown to be affected by the chemical form of N in the soil (Maestri et al. 2010). However, there are other aspects to the soil environment than just inorganic and organic chemicals; namely dominant biota, including rhizobacteria and mycorrhizae.

12.3.3 *Rhizobacteria and Mycorrhizae*

The importance and potential role of rhizobacteria and mycorrhizae in the uptake of heavy metals from the soil is a vast and largely unexplored area of research, but it could greatly aid in the development of phytoremediation technologies. Rhizobacteria and mycorrhizae, through metabolic activities, affect the soil environment, including bioavailability and mobility of elements (Mench et al. 2009). Abou-Shanab et al. (2003) found that rhizobacteria play an important role in increasing availability of Ni in soil, thereby enhancing Ni accumulation by *A. murale*. They isolated three bacteria species (*Sphingomonas macrogoltabidus*, *Microbacterium liquefaciens*, and *M. arabinogalactanolyticum*) from the rhizosphere of *A. murale* and added them to sterile and non-sterile Ni-rich serpentine soils, to examine their ability to solubilize Ni in soil and their effect on Ni uptake by *Alyssum*. The authors observed that *S. macrogoltabidus* significantly reduced Ni extraction from soil; *M. arabinogalactanolyticum* significantly increased Ni extraction from soil; and *M. liquefaciens* had no effect. They also found that inoculation with these bacteria did not significantly impact extractability of other metals. However, it was reported that Ni uptake into the shoot of *A. murale* increased by 17%, 24%, and 32.4% for *S. macrogoltabidus*, *M. liquefaciens*, and *M. arabinogalactanolyticum*, respectively. Whiting et al. (2001) conducted a study on rhizospheric bacteria and their role in Zn uptake and concluded that microbes play an important role in Zn uptake of *T. caerulescens*. Addition of randomly selected strains of bacteria (*M. saperdae*, *Pseudomona monteilly*, and *Enterbacter*

cancerogenes) to surface-sterilized seeds of *T. caeruleus* increased Zn shoot concentration two-fold compared to the sterile control and total Zn accumulation increased four-fold along with shoot biomass, although not with root biomass (Whiting et al. 2001). Interestingly, the same experiment conducted with non-hyperaccumulator *T. arvense* had no effect. The authors concluded that bacteria increased availability of water-soluble Zn; liquid media that supported bacterial growth mobilized 1.2- to 1.8-fold more Zn from soil. Therefore, the overall conclusion of the study was that bacteria facilitate solubilization of nonlabile forms of Zn (*i.e.* increase availability of water-soluble Zn) in the rhizosphere of hyperaccumulator *T. caeruleus*.

Of course, when heavy metals are present at toxic levels, all soil biota are affected, which means that the plant-soil relationship will also be determined by the level and type of contamination. Zarei et al. (2010) conducted a study on the impact of metal contamination on arbuscular mycorrhizal fungi (AMF). These are common soil organisms that have been repeatedly found in heavy metal contaminated soils (Del Val et al. 1999; Khan 2005; Giasson et al. 2006). AMF have widespread symbiotic relationships with plants, but the specific nature of these relationships varies with specific AMF isolates, host plants, and soil properties (Zarei et al. 2010). AMF were examined at an open pit mine in Iran, in soils ranging from non-polluted to Pb- and Zn-polluted, and involving several dominant plant species. With increasing Pb and Zn concentrations, the number of AMF sequence types decreased, suggesting a negative correlation between heavy metal contamination and AMF diversity and abundance; however, one sequence type was found only in the highly contaminated area, indicating a locally adapted strain of AMF (Zarei et al. 2010). The authors also found that CaCO₃ and available P were important in AMF distribution, highlighting the need to take the edaphic environment into careful consideration when planning phytoremediation trails.

12.4 Implications for Phytoremediation and Next Steps

The widespread application of phytoremediation will have many challenges beyond those encountered in the laboratory (Angle and Linacre 2005). There are myriad of site-specific factors which will influence the success of any phytoremediation effort. Practical application of phytoremediation technologies will necessarily be informed by laboratory experiments.

The success of any phytoremediation effort is determined by several key factors. Many hyperaccumulators are metal-specific, can only be used in their native habitats, have slow growth rates and shallow root systems as well as low biomass, and not every metal of interest has a corresponding hyperaccumulator (Schat et al. 2000; Pilon-Smits 2005; Rascio and Navari-Izzo 2011). There is also almost no research to date on agronomics and disease potential, and only limited testing of genetics and breeding potential (Rascio and Navari-Izzo 2011). Additionally, the

key roles of the rhizosphere in metal tolerance and hyperaccumulation should be further investigated (see Alford et al. 2010).

According to Chaney et al. (2007), development of phytoremediation technologies rely on two major criteria: domestication of hyperaccumulators and cloning of all relevant genes. Domestication of hyperaccumulator species has already been shown effective for hyperaccumulators of Ni, Cd, Se, and As (Chaney et al. 2007) and there are many ongoing efforts exploring the genetic basis and cloning of relevant genes (Yang et al. 2005a, b; Pilon-Smits and LeDuc 2009; Wright and von Wettberg 2009).

Domestication of plants implies cultivation. When plants are cultivated in the field, it is necessary to think about length of season, climate, level of contamination, method of seed-sowing (spacing, etc.), and plant-soil interactions not only at the level of metal-availability, but also in terms of fertility, so that optimum above-ground biomass can be achieved. For example, Baker et al. (2000) found that *Thlaspi* and *Alyssum* species were responsive to soil fertilization and that growth potential can be enhanced by fertilization. This, however, raises the question of the types of fertilizer being used—a commercial chemical fertilizer, compost, seaweed, or some other organic treatment. All provide nutrients to the plant, but chemical fertilizers do not necessarily enhance the quality of the soil. One experiment to assess the feasibility of Ni phytomining tested *A. bertolonii* in the field with applications of N-P-K fertilizer over 2 years, resulting in increasing biomass three-fold- or about 13,500 kg ha⁻¹ without diluting the shoot Ni concentration of the plants (Rascio and Navari-Izzo 2011). A similar field experiment with the South African Ni-hyperaccumulator *Berheya coddii* (Asteraceae) reported a fertilized yield of 22,000 kg ha⁻¹ with an estimated amount of 100 kg Ni ha⁻¹ being achievable at most sites worldwide (Rascio and Navari-Izzo 2011). However, further research is needed to determine if and when fertilizer should be used, the degree to which it increases growth potential, and what after-effects it might have of the remediated site, positive or negative. It is also important to keep pH in mind, and optimum pH for phytoextraction may need to be determined for individual soils as well as plant species to be utilized (Chaney et al. 2007).

Wang et al. (2006) found that *T. caerulescens*, when soil pH was optimized, accumulated 40% of total Cd in highly contaminated soil and 36% in low-contamination soils, with just one planting. This suggests that finding optimum pH that is site- and plant-specific could prove very useful in maximizing phytoremediation potential. Further, *T. caerulescens* hyperaccumulates Cd and Zn, and yields a maximum of 2 tons ha⁻¹ of shoot dry matter (Rascio and Navari-Izzo 2011). However, pot and field studies have shown that *T. caerulescens* can, with selective breeding, achieve the equivalent of a 5 ton ha⁻¹ growth rate with a desirable combination of yield and shoot metal concentration (Rascio and Navari-Izzo 2011).

Another important factor to consider in applied phytoremediation is biomass potential. The greater the above-ground biomass, the greater the concentration of metal to be removed. Salt et al. (2000) found that the non-accumulator *T. arvense* displayed lower shoot biomass than its hyperaccumulating congener, *T. goesingense*, after Ni exposure. However, both plants translocated Ni to the

shoot at the same rate when exposed to nontoxic Ni levels (Salt et al. 2000). Wenzel et al. (2003) showed that mean shoot biomass of *T. goesingense* exceeded excluders' biomass (*S. vulgaris* and *R. acetosella*), and furthermore that root biomass decreased in the order of *S. vulgaris* > *T. goesingense* > *R. acetosella*. This gives credence to the previously discussed phenomenon of excluders having greater root biomass than hyperaccumulators.

In the context with the problem of contamination by multiple metals, several *Thlaspi* species can accumulate more than one metal (Pongrac et al. 2009), notably *T. caerulescens*, which in addition to Zn and Cd can also accumulate Ni and Pb, and could remove up to 60 kg Zn ha⁻¹ and 8.4 kg Cd ha⁻¹ (Rascio and Navari-Izzo 2011). However, a major sticking point remains the time frame, which can be decades. It is estimated that nine croppings of *T. caerulescens* would be required to remediate soil from 440 to 300 mg Zn kg⁻¹, and that to use this plant on soils containing 2,100 mg Zn kg⁻¹ would take 28 years (Rascio and Navari-Izzo 2011). Therefore, the level of contamination always needs to be taken into account. Further, levels of hyperaccumulation vary among species and among populations and ecotypes of the same species. For example, among ecotypes of *T. caerulescens*, a southern French ecotype accumulates more Cd than Zn (Rascio and Navari-Izzo 2011) most effectively at an acidic pH and, importantly, in useful amounts (Chaney et al. 2007). Thus, extensive field explorations are required to locate and characterize population-level differences in the metal-accumulating potential of known metal accumulating species as well discover new accumulators, especially from the Brassicaceae, a family with a predisposition for metal tolerance and accumulation.

Chaney et al. (2008) also point out that serpentine soils, because of their origins from peridotite and related parental rocks, tend to have low Ca/Mg quotients. Soil Ca has been shown to reduce Ni uptake (and phytotoxicity) in crop plants, so plants native to serpentine soils may have limited tolerance for soils used for commercial phytomining. The authors compared two serpentine endemic species, *A. murale* and *A. pintodasilvae*, with cabbage (*Brassica oleracea*) in nutrient solutions that mimicked serpentine as well as normal soils, with varying levels of Ca and Mg. The *Alyssum* taxa showed much greater tolerance to high Ni and Mg and low Ca than cabbage. A non-linear correlation was found between Ni translocation and shoot concentration, shoot yield, and shoot Ca levels; all increased with increasing Ca up to 2 mM, then decreased at the highest Ca levels. The results suggest that shoot biomass and yield potential when phytomining with *Alyssum* taxa in high-Mg soils would benefit from Ca application (Chaney et al. 2008).

Many laboratory experiments rely heavily on the application of synthetic chelators, such as EDTA, to make metals more available to plants. However, as Chaney et al. (2007) argue, this approach is impractical, and indeed counterproductive, in the field. EDTA and similar synthetic chelators contaminate the soil themselves, thus negating the intended purpose of soil cleanup. EDTA increases metal uptake in plants by injuring the roots of plants, thus decreasing the barriers to uptake. Further, only a fraction of the metal "freed up" by EDTA is taken up into the plant shoot, leaving the rest to be leached out of the topsoil. Add to the mix that

EDTA is also expensive, especially when used in field trials, and you have a strong case against the practical use of synthetic chelators (Chaney et al. 2007). According to Chaney et al. (2007), too much research has focused on trying to develop Pb extraction technologies with *B. juncea* (Podar et al. 2004; Lai et al. 2008; Hanen et al. 2010; Zarei et al. 2010), whereas in reality, even if there was enough phosphate present in contaminated soils to give good crop yields, most Pb remains in the soil or the roots and, without soil amendments, *B. juncea* has little ability to absorb Pb from contaminated soils (Chaney et al. 2007).

There are, however, promising examples of phytoremediation and phytomining in the field. Chaney et al. (2007) point out that *Alyssum* biomass is one of the richest known Ni bio-ores, and that *Alyssum* species can be effectively developed into commercial phytomining practices. Broadhurst et al. (2004) report that *A. murale* and *A. corsicum*, two species that are endemic to serpentine soils in Mediterranean Europe, have been employed in commercial phytomining of Ni because, unlike many other serpentine endemic species, they can hyperaccumulate Ni from other types of soil, including limestone, organic, and loam. *Alyssum bertolonii* and *Berkheya coddii* are fast-growing Ni hyperaccumulators that have been shown in small-scale field experiments to be suitable for phytoremediation, especially as they have high-biomass, high-Ni concentration, and are easy-to-propagate; further, *B. coddii* is cold-tolerant making it useable even in temperate regions (Rascio and Navari-Izzo 2011). Experiments with gene transfer have also shown that transgenic *B. juncea* takes up more Se and has greater Se tolerance than the wild species when grown under both soil and hydroponic conditions (Rascio and Navari-Izzo 2011). Somatic hybrids have been produced between *T. caerulescens* and *Brassica napus* which accumulated levels of Zn that would have been toxic to *B. napus* at a greater biomass than *T. caerulescens* (Rascio and Navari-Izzo 2011). Similar hybrids have been made between *T. caerulescens* and *B. juncea* which removed large amounts of Pb from soil (Rascio and Navari-Izzo 2011). A transgenic *B. juncea* plant was tested by Banuelos et al. (2005) as well as Bennett et al. (2003) under field conditions and found to have greater biomass and Se (Banuelos et al. 2005) and Cd (Bennett et al. 2003) accumulation potential than the wild type. These examples make a case for continuing research into hybridization, selective breeding, and gene transfer and controlled field trials of 'engineered' taxa prior to widespread use (Eapen and D'Souza 2005).

One caveat when considering phytoremediation or phytomining is the environmental impact of introducing hyperaccumulating species to a non-native habitat. As in a recent case in O'Brien, Oregon, USA, *A. murale* and *A. corsicum* from Mediterranean Europe, appear to have naturalized and become invasive in nearby serpentine outcrops, potentially threatening native plants (<http://www.oregon.gov/ODA/PLANT/WEEDS/edrr.shtml>). It is unclear how invasive these plants would be in the long-term or their impact beyond the immediate vicinity; however, it is vital to understand the biology and ecology of the plant to be used, in as much detail as possible before undertaking field-based phytoremediation or phytomining operations.

12.5 Conclusions

In this chapter, we have discussed the major components of internal plant physiology and plant-rhizosphere interactions critical for understanding phytoremediation. Much of this knowledge is based on studies conducted on members of the Brassicaceae. The *Brassica* oilseeds and related species (notably, *Alyssum* and *Thlaspi*) will continue to lead the way for developing effective phytoremediation technologies. Additionally, the serpentine endemic species *Streptanthus polygaloides* (Brassicaceae) has also shown promise as a viable candidate for phytoremediation and phytomining of Ni (Boyd and Davis 2001; Li et al. 2003). We have also discussed areas of plant and soil properties that require further research as phytoremediation becomes a more viable and desirable method of environmental cleanup, as well as findings from recent applied phytoremediation studies. *Brassica* oilseeds represent a large proportion of heavy metal hyperaccumulating plants and, with an ever-growing understanding of the mechanisms by which they hyperaccumulate metals and the optimum conditions under which they do so, comes an ever-increasing commercial expansion of phytoremediation—a much less expensive and more environment-friendly form of soil-contaminant cleanup than traditional methods of excavation and chemical treatment.

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